

Sea lice (Lepeophtheirus salmonis) and anchor worms (Lernaea cyprinacea) found on sea trout (Salmo trutta) in the River Minho catchment, an important area for conservation in NW Spain.

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ABSTRACT

1. The International Stretch of the River Minho (ISRM), in NW Spain, is an important area for marine and freshwater conservation. It constitutes the southern limit of distribution of migratory sea trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*), where their populations are considered as vulnerable and endangered, respectively.
2. A sample of sea trout from the River Minho catchment (NW Spain) was examined for ectoparasites.
3. Sea lice (*Lepeoptheirus salmonis*) were found on 10/113 fish (9%). Infection levels ranged from 0–8 lice/fish. Anchor worms (*Lernaea cyprinacea*) were also found, on three fish (3%).
4. Lice identifications were confirmed by taxonomic and molecular analysis. This is the first time the presence of *L. salmonis* has been confirmed in NW Iberia.
5. The confirmed presence of these parasites, will inform conservation agencies, wild fisheries and sustainable aquaculture initiatives in this important area.

KEY WORDS:

Lepeoptheirus, *Lernaea*, *Salmo*, River Minho, NW Spain

INTRODUCTION

The River Tea is a major tributary (length c. 50 km) of the International Stretch of the River Minho (ISRM), which flows along the natural frontier between Galicia (Spain) and Portugal and drains into the Atlantic Ocean. The lower Minho is also designated SCI (Site of Conservation Interest) and SPA (Special Protected Area), both included in the EU Natura 2000 network. With c. 43 km (85%) of its length available for migration, the River Tea is considered to provide the greatest carrying capacity for migratory species in the entire River Minho catchment. The presence of a major hydroelectric dam at 76 km upstream of the mouth of the River Minho, currently restricts fish migration to the River Tea and a few other tributaries in the Lower reaches of the ISRM.

The River Minho catchment, along with the River Lima, constitutes the current southern limit of the geographic distribution of migratory sea trout (*Salmo trutta* L) and Atlantic salmon (*Salmo salar* L) in Europe (Caballero, 2013). Historically, it supported an important salmon population, which has been detrimentally impacted by the hydroelectric dam and other anthropogenic factors (Sousa *et al.*, 2008). Currently, the Atlantic salmon is listed as *endangered* in the ISRM and as *vulnerable* in Galician Rivers. Sea trout currently maintain better stock levels and distribution, and a local fishery is maintained, although it is also listed as *vulnerable* in ISRM and Galician Rivers (Migranet, 2012). Sea trout has two migration periods in Galician rivers, in spring-summer and October onwards. These are influenced by a number of factors, including water temperature, tides and high river flows associated with autumnal precipitation (Caballero, 2013).

Sea lice (Copepoda: Caligidae) commonly parasitize wild and farmed salmonids (Kabata, 1979). The most important species in the North Atlantic region, *Lepeoptheirus salmonis* Krøyer, is a host-specialist that requires a suitable, salmonid host for settlement and survival (Kabata, 1979; Boxaspen, 2006; Costello, 2006; Torrisen *et al.*, 2013). Sea lice are the most pathogenic parasites

in salmon aquaculture that may cause important economic losses (Costello 2009a) and has the potential to seriously impact wild salmonid populations (Boxaspen, 2006; Costello, 2006; Fraser, 2008; Costello, 2009b; Middlemas *et al.*, 2010, 2013; Jackson *et al.*, 2013; Torrissen *et al.*, 2013; Serra-Llinares *et al.*, 2014; Gjellan *et al.*, 2014; Skaala *et al.*, 2014). Sea lice can cause significant physiological and pathological consequences on salmonid hosts affecting their growth, fecundity, food-conversion efficiency, reduced appetite and survival due to their feeding behaviour, which erodes mucus, skin and underlying tissues, thus can also causing secondary infections (Boxaspen, 2006; Costello, 2006; Torrissen *et al.*, 2013). Moreover, host behaviour can be altered as a response to sea lice infection resulting in premature return of sea trout which reduces growth and reproductive potential (Gjelland *et al.*, 2014). Sea lice infection may also be an important contributor to mortality of anadromous trout that appears to be directly related to salmon farming and production of salmon lice on farmed fish (Skaala *et al.*, 2014).

Anchor worms (*Lernaea cyprinacea*) (Copepoda: Lernaeidae) are widely distributed, freshwater parasites that can also seriously impact wild and farmed host fish stocks (Sánchez-Hernández, 2011). In Spain, it is considered to be an exotic, invasive species; which may have been introduced by importing infected common carp (*Cyprinus carpio*) from Asia (García-Berthou *et al.*, 2007). *Lernaea cyprinacea* is now well established in a number of rivers, and has recently been recorded in wild populations of non-migratory brown trout (*S. trutta*) in central Spain (Sánchez-Hernández, 2011), but not previously in sea trout.

In terms of the conservation status and economic importance of the ISRM, and the fact that proposals to establish salmon farming in this area are under serious consideration, it is important to determine if *L. salmonis* occurs naturally in in this area, and in NW Iberia in general. The presence of sea lice in Galicia, on sea trout in the River Ulla, was previously reported (Caballero, 2013); although no information on identification methodology was provided, and *L. salmonis* has,

to date, not been confirmed by taxonomic or molecular identification. *Lernaea cyprinacea* is an invasive species in Iberian freshwater systems (Sánchez-Hernández, 2011) that has not, to date, been reported on migratory fish.

The following study was initiated, therefore, to determine the occurrence of *L. salmonis*, and *L. cyprinacea* on migratory salmonids in the River Minho, in order to inform future conservation management strategies for the ISRM.

METHODS

A total of 113 mature sea trout (*sample 1*) was captured with a catch net at the *A Freixa* sampling station on the River Tea (map reference: UTM29T X540116 Y4670698) during annual, upstream spawning migrations, in October 2013. *A Freixa* is located c.50 km from the marine environment. During December 2013 to May 2014, 279 post-spawning fish, moving downstream, were also captured and examined (*sample 2*). The fish were initially anesthetized in a tank of freshwater containing phenoxyethanol (dose: 2 cc/10 L [200 ppm]), measured (weight [g], length [mm]), and then examined for sea lice. The number, species and sex of lice found on each fish were noted. The distribution of individual lice on the host body surface was also recorded, using a standard technique (Treasurer and Bravo, 2011). Attached lice were then carefully removed and stored in 70% ethanol for further taxonomic investigation and molecular identification. Anchor worms were also noted and stored, similarly.

Specimens of *L. salmonis* were examined with a low-power stereo microscope and identified using standard taxonomic keys (Kabata, 1979, 1992). Taxonomic diagnosis of *L. salmonis* was later confirmed by molecular analysis. Genomic DNA from two specimens was isolated using MACHEREY-NAGEL NucleoSpin®Tissue kit following manufacturer recommended protocols. The entire 18S (18S rRNA) was amplified using the forward primer 18SU467F (5'ATC CAA

GGA AGG CAG CAG GC 3') and reverse primer 18SL1310R (5'CTC CAC CAA CTA AGA ACG ACG 3'). PCR reactions were carried out in a total volume of 25 µl containing 100 ng of genomic DNA, 10 µM of each primer, 2.5 µl of 10x buffer, 0.5 µl of dNTPs and 5 U/µl of Taq DNA polymerase (From *Thermus Aquaticus* BM, recombinant, Roche). PCR cycling parameters included denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min. PCR products were purified using illustra ExoStar 1-Step following manufacturer recommended protocols, with some modifications. We added 4 µl of reactive illustra ExoStar 1-Step and incubated the mix for 15 min at 37 °C. For inactivation of the reactive added we incubate the mix 20 min at 80 °C. Samples with DNA concentration in clean reaction of 20 ng/µl were sequenced by SECUGEN®.

RESULTS

Sample 1: Attached ectoparasites (sea lice and anchor worms) were found on some of the fish (*S. trutta*) migrating upstream, in October 2013. Recorded host fish size data, and infection levels of sea lice are provided in Table 1. Overall, 26 male and 87 female fish, migrating upstream, were captured and examined. Attached lice were observed on 10 fish (1 male + 9 females), representing an overall prevalence of c. 9% fish infected (Table 1). All lice specimens found were identified as the salmon louse, *Lepeoptheirus salmonis*. Individual infection levels were low, ranging from 1–8 lice/fish. Totals of 26 male and 7 female *L. salmonis* were recorded (Table 2). Only mature lice were observed, although no egg strings were found attached to the female lice.

Observed distributions of *L. salmonis* on the host body surface are shown in Table 2. Skin lesions in the posteroanal region, caused by attached lice, were also observed on three fish (R-19, R-25, R-59).

Taxonomic identification of *L. salmonis* was later confirmed by molecular analysis. Observed Blast values of 100% indicated that all the lice found attached to the fish in the River Tea sample were *L. salmonis*. Sequences deposited in *GenBank* (Accession Nos: KM047082, KM04783) correspond to *L. salmonis* specimens collected from host fish (*S. trutta*) R-100.

Parasitic anchor worms (Copepoda: Lernaeidae) were also found, attached to the anterodorsal regions of three fish (R-43, R-50, R-79). These were collected and later identified as *Lernaea cyprinacea* L (K. Mackenzie, pers. comm.).

Sample 2: No parasites were found on the fish ($N = 279$) migrating downstream, that were captured and examined at the station, 2–7 months later (December 2013 to May 2014).

DISCUSSION

The survival of *L. salmonis* in freshwater is clearly compromised (Bricknell *et al.*, 2006; Connors *et al.*, 2008; Torrissen *et al.*, 2013), although their endurance in different river systems is not well known. The majority of attached lice normally die rapidly when the host fish enters freshwater, depending on host species and size. Adult *L. salmonis* can tolerate intermediate salinities, during the transition of host fish from seawater to freshwater, for a short period of time (Hahnenkamp & Fyhn, 1985; Bricknell *et al.*, 2006; Connors *et al.*, 2008). To date, there is no scientific information on the survival of attached lice on sea trout migrating upstream. Our findings indicate that *L. salmonis* can tolerate upstream migrations of at least 50 km. The absence of lice in post-spawning fish migrating downstream, however, indicate that *L. salmonis* cannot tolerate prolonged periods in freshwater. The lack of juvenile lice stages or eggs at *A Freixa* indicates that the host fish had already been in freshwater for a few days prior to capture, since juvenile *L. salmonis* cannot survive for long in freshwater, and gravid females are known to quickly become stressed, causing them to

eject their egg strings (McLean *et al.*, 1990). The presence of attached sea lice c.50 km upstream therefore indicates a rapid migration of *S. trutta* from the marine environment.

Previous, unconfirmed historical records at A Freixa sampling station indicate that sea lice are often found on migratory *S. trutta* and *S. salar* at this location (unpublished data). This is the first time that the natural occurrence of *L. salmonis* in the rivers of NW Iberia, representing the extreme, southern geographical limit of its salmonid host, has been confirmed. Sea lice infections can cause high mortalities in wild and farmed salmonid populations, threatening wild stocks and associated fisheries, and resulting in significant additional costs to the aquaculture industry (Costello, 2006, 2009a; Jackson *et al.*, 2013, Torrisen *et al.*, 2013). Enhanced transmission of sea lice, from wild to farmed fish hosts and vice versa, may seriously impact the conservation status of resident salmonid populations in some localities (Frazer, 2008; Costello, 2009b; Middlemas *et al.*, 2010, Serra-Llinares *et al.*, 2014; Gjellan *et al.*, 2014; Skaala *et al.*, 2014).

In other areas in the geographic range of *L. salmonis*, high infection rates and numbers of lice on wild sea trout have been linked to local salmonid farming operations (Middlemas *et al.*, 2010; 2013, Serra-Llinares *et al.*, 2014). Middlemas *et al.* (2010) found sea lice prevalence of 47.5% on wild trout smolt caught at river mouths on the west coast of Scotland. They also investigated the relationship between salmon farming and infestations of sea lice on wild sea trout and found that 52.5% of fish sampled were infected during 2003 to 2009 (Middlemas *et al.*, 2013). Serra-Llinares *et al.* (2014) reported a higher prevalence of lice in wild sea trout caught within 30 km of a salmon farm in Norwegian waters, compared to those caught at distances >30 km. Skaala *et al.* (2014) reported prevalences of 100% in the Hardangerjord, a region with a high density of salmon farms of Western Norway.

Atlantic salmon (*S. salar*) and Coho salmon (*Oncorhynchus kisutch* Walbaum) were previously farmed in Galician waters, during 1976–2005. Production ceased in 2005, owing to a number of operational difficulties. In 2011, however, experimental cages were re-installed and *S. salar* was again farmed in Galician waters for a brief period. Operations have since ceased, but economic and political interest in re-establishing a salmon farming industry in Galicia remains high (Casal, 2013).

Thus, the confirmed presence of *L. salmonis* and *L. cyprinacea* in Galician river systems should be considered in order to inform conservation managers and possible future salmonid farming proposals. The conservation status of endangered Atlantic salmon and vulnerable sea trout populations in the ISRM and other river systems in the NW Iberian peninsula could potentially be affected by inappropriately designed fish farming operations at sensitive locations (for information on this subject, see Middlemas *et al.*, 2013). Further studies of salmon lice epidemiology at the river mouth are required, in order to confirm whether or not infection takes place there, survival of salmon louse during transition from seawater to freshwater and parasite infection levels in wild upstream spawning salmonids and smolts at the beginning of their seaward migration. *Lernaea cyprinacea* can also seriously impact wild fisheries and freshwater aquaculture operations (Piasecki *et al.*, 2004), and its presence in migratory salmonids in the Iberian Peninsula may also be significant.

Since *L. salmonis* (and its migratory fish hosts) are at the extreme southern limit of their geographic distribution, any local effects of climate change (e.g. elevated water temperatures) on the occurrence of parasite and salmonid host in the River Minho system are likely to be significant. The occurrence of *L. cyprinacea* in this area might be enhanced by climate change, since this species can parasitize a large number of alternative fish hosts, and is also probably more tolerant of higher water temperatures. Environmental change has the potential to affect complex host-

parasite relationships and the efficacy of treatments in aquaculture operations in a number of ways (Callaway *et al.*, 2012).

Further investigations are required, in order to determine the prevalence of sea lice, anchor worms and other parasites in the migratory salmonid stocks of NW Iberia. Knowledge and understanding of how they interact with their hosts in the riverine and marine environments will be crucial for the conservation and management of sustainable wild fisheries and aquaculture operations in this important area.

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Table 1. Host fish size (length/weight) data, and prevalence of attached sea lice observed, in a sample of anadromous *S. trutta* from the River Tea (Minho catchment, NW Spain).

Sex	N	Length (mm)		N	Weight (g)		Prevalence		Lice loads	
		Mean (\pm SD)	Range		Mean (\pm SD)	Range	N ¹	(%)	Abundance (\pm SD)	Intensity (\pm SD)
M	26	444 (\pm 80.9)	347–636	21	1059 (\pm 664.4)	525–3376	1	(3.8)	0.27 (\pm 1.37)	7 -----
F	87	402 (\pm 54.6)	320–638	81	754 (\pm 450.0)	330–3534	9	(10.3)	0.30 (\pm 1.20)	2.89 (\pm 2.67)
All	113	412 (\pm 63.8)	320–638	113	-----	330–3534	10	(8.8)	0.29 (\pm 1.24)	3.30 (\pm 2.83)

1. Number of fish infected with sea lice.

Table 2. Individual host fish details, sea lice distributions and overall lice loads of infected anadromous *S. trutta* specimens observed in the River Tea, NW Spain.

Host fish details				Distribution of lice							Overall	
Spec		L	W	N/region ¹							N lice	
ID	Sex	(mm)	(g)	HD	AD	PD	AV	PV	PA	CD	Mal	Fem
R-3	F	435	889	0	0	0	1	0	0	0	1	0
R-10	F	367	557	1	0	0	0	0	0	0	1	0
R-11	F	336	400	1	0	0	0	0	0	0	1	0
R-19	F	397	532	0	0	1	0	0	1	0	1	1
R-24	F	444	838	2	1	4	0	0	0	0	7	0
R-25	F	362	506	0	0	1	0	0	1	0	1	1
R-31	F	430	796	0	2	0	0	0	0	0	1	1
R-59	F	355	439	0	1	0	0	0	1	0	1	1
R-92	M	445	1052	3	2	2	0	0	0	0	6	1
R-100	F	433	-----	3	0	3	0	0	2	0	6	2

1. Codes for host surface regions followed Treasurer & Bravo (2011): HD = head, AD = anterior dorsal, PD = posterior dorsal, AV = anterior ventral, PV = posterior ventral, PA = post anal, CD = caudal.